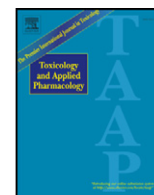
Toxicology and Applied Pharmacology 280 (2014) 30–35

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap



High Impact Short Article

Evaluation of the usefulness of novel biomarkers for drug-induced acute kidney injury in beagle dogs

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ARTICLE INFO

Article history:
Received 23 January 2014
Revised 27 June 2014
Accepted 3 July 2014
Available online 14 July 2014

Keywords:
Beagle dogs
Biomarkers
Nephrotoxicity
Preclinical study
Neutrophil gelatinase-associated lipocalin
Clusterin

ABSTRACT

As kidney is a major target organ affected by drug toxicity, early detection of renal injury is critical in preclinical drug development. In past decades, a series of novel biomarkers of drug-induced nephrotoxicity were discovered and verified in rats. However, limited data regarding the performance of novel biomarkers in non-rodent species are publicly available. To increase the applicability of these biomarkers, we evaluated the performance of 4 urinary biomarkers including neutrophil gelatinase-associated lipocalin (NGAL), clusterin, total protein, and N-acetyl-β-D-glucosaminidase (NAG), relative to histopathology and traditional clinical chemistry in beagle dogs with acute kidney injury (AKI) induced by gentamicin. The results showed that urinary NGAL and clusterin levels were significantly elevated in dogs on days 1 and 3 after administration of gentamicin, respectively. Gene expression analysis further provided mechanistic evidence to support that NGAL and clusterin are potential biomarkers for the early assessment of drug-induced renal damage. Furthermore, the high area (both AUCs = 1.000) under receiver operator characteristics (ROC) curve also indicated that NGAL and clusterin were the most sensitive biomarkers for detection of gentamicin-induced renal proximal tubular toxicity. Our results also suggested that NAG may be used in routine toxicity testing due to its sensitivity and robustness for detection of tissue injury. The present data will provide insights into the preclinical use of these biomarkers for detection of drug-induced AKI in non-rodent species.

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Introduction

Kidney is a major target organ affected by exogenous toxicants. Nephrotoxicity is a kidney-specific feature, in which excretion is disrupted due to exposure to toxic chemicals or drugs. Recently, drug-induced nephrotoxicity has been reported to contribute to approximately 20% of cases of hospital-acquired acute kidney injury (AKI) (Luyckx and Naicker, 2008). AKI is characterized by failure of the kidneys to meet the excretory, metabolic, and endocrine demands of the body. Because these injuries may be mild and go unnoticed and it lacks a consensus definition of AKI severity, the early identification of AKI remains difficult (Abelha et al., 2009; Bellomo et al., 2004). Therefore, there is a great need for sensitive and specific markers for early identification of nephrotoxicity during drug research and development.

To date, blood urea nitrogen (BUN) and creatinine (Cr) are most commonly used biomarkers of nephrotoxicity (Hosten, 1990). However, both of these biomarkers are known to have low sensitivity for detection, and it is therefore important to identify novel biomarkers with higher sensitivities in order to assure the diagnosis of nephrotoxicity, especially during the preclinical stage of new drug development (Lisowska-Myjak, 2010; Martensson et al., 2012). In past decades, biotechnological advances, particularly omics technologies, have provided efficient methods for the discovery of novel mechanism-based biomarkers for target organ toxicities. A series of novel candidate biomarkers of drug-induced nephrotoxicity were discovered (Amin et al., 2004; Goodsaid, 2004; Thukral et al., 2005; Wang et al., 2008). In 2009, the Food and Drug Administration (FDA) of the United States and European Medicines Agency (EMEA) endorsed the use of a number of these urinary biomarkers for the detection of AKI in the context of nonclinical drug development, including urinary total protein (uTP), kidney injury molecule-1 (KIM-1), clusterin, β2-microglobulin (B2M), cystatin-c (CysC), trefoil factor 3, and albumin (ALB) (EMEA, 2009). N-acetyl-β-D-glucosaminidase (NAG), a proximal tubule lysosomal enzyme, has been extensively studied as a sensitive, persistent, and robust indicator of tubular injury (Bourbouze et al., 1984; Fujita et al., 2002; Vaidya et al., 2008). Furthermore, neutrophil gelatinase-associated lipocalin (NGAL) has recently been recognized as a novel candidate nephrotoxic

Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; NAG, N-acetyl-β-D-glucosaminidase; AKI, acute kidney injury; ROC, receiver operator characteristics; BUN, blood urea nitrogen; Cr, creatinine; uTP, urinary total protein; ELISA, enzyme-linked immunosorbent assay; AUC, area under the curve; CI, confidence interval.

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http://dx.doi.org/10.1016/j.taap.2014.07.002
0041-008X/© 2014 Published by Elsevier Inc.

biomarker (Akrawinthawong et al., 2013; Haase et al., 2011; McWilliam et al., 2012; Peacock et al., 2013; Tonomura et al., 2010).

The overall aim of these novel biomarkers is to enhance the safety profiles of drugs by achieving earlier detection of nephrotoxicity in preclinical studies and better translatability into humans. Additionally, early detection of nephrotoxicity is very helpful for the early strategic decision in drug research and development. Preclinical safety studies are designed to identify pharmacological and toxicological effects of test articles prior to initiation of human studies and throughout clinical development. Generally, animal testing in drug development studies requires at least 2 species, most typically rats and dogs, although non-human primates and guinea pigs are also commonly used. Many previous studies have used rodent-based animal models for evaluating the effectiveness of novel renal biomarkers for improved detection of drug-induced kidney injury (Com et al., 2012; Hoffmann et al., 2010a; Hoffmann et al., 2010b; Sieber et al., 2009; Tonomura et al., 2010; Wang et al., 2008). However, limited data regarding the performance of novel biomarkers in non-rodent species are publicly available.

Therefore, in order to provide a better understanding of the usefulness of these potential biomarkers in non-rodent species, we comprehensively evaluated the performance of 4 urinary biomarkers, including NGAL, clusterin, NAG, and uTP, relative to histopathology and routine clinical chemistry parameters in the model of acute kidney injury induced by gentamicin in beagle dogs.

Materials and methods

Animals and maintenance. Male beagle dogs (5–6 months of age) were purchased from Beijing Marshall Biotechnology Co., Ltd. (China) and maintained in stainless steel cages in a room equipped with an air-filtering system. Food was provided twice a day at 250 ± 10 g/animal/day, and water was offered ad libitum. Food and water were removed during urine collection. Each dog was individually housed and identified by tattoo and animal number. Animals were quarantined for 14 days before the study was conducted. Animals were accepted for use in the study based on body weight and physical examinations performed during the quarantine period. All animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the National Center for Safety Evaluation of Drugs (NCSED), Beijing, China.

Study design. This study included 2 independent animal experiments. In the first experiment, a total of 12 dogs were randomized into 2 groups: the control group ($n = 3$) and the gentamicin-treated group ($n = 9$). Dogs were treated by intramuscular injection with 80 mg/kg gentamicin (AppliChem, USA) each day for 9 days. Dogs in the control group were treated with the same volume of saline. Three dogs in the treated group were respectively euthanized on study days 3, 6, and 9, while the 3 dogs in the control group were euthanized on day 9. All animals were observed every day. Urine samples were collected on ice from 09:00 to 15:00 during the quarantine period (day −3) and on days 1, 3, 6, and 9. Urine samples were centrifuged at 1500 rpm for 5 min, and multiple aliquots of urinary supernatants were immediately frozen at 70 °C until analyzed by immunoassay for detection of novel biomarkers as described. Blood samples were collected after urine collection during the quarantine period (day −3) and on days 3, 6, and 9. Sera were separated for serum Cr and BUN analysis, and the remaining sera were divided in aliquots and stored at −70 °C until use. The kidneys of all experimental animals were collected for histopathological examination. In the second experiment, we used a greater number of dogs ($n = 5$ for the control group and $n = 15$ for the treated group, with 5 animals in the treated group euthanized on study days 3, 6, and 9); all other treatment conditions remained the same. Furthermore, cortex tissues were collected from the left kidneys of all animals in the second experiment, which were used for mRNA expression analysis of new biomarkers.

Urinalysis and blood chemistry examinations. Urine Cr (Wako, Japan), uTP (Roche, Switzerland), and NAG (Wako, Japan) in the urine supernatant as well as serum Cr and BUN (Wako, Japan) were determined using a 7060 automatic biochemistry analyzer (Hitachi, Japan). Urinary clusterin (Biovender, Czech) and NGAL (Bioporto, Denmark) levels in urine supernatants were detected using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. The limits of detection for clusterin and NGAL were 0.2 ng/mL and 0.56 pg/mL, respectively. Concentrations of urinary biomarkers were normalized to urinary Cr.

Pathological examination. At necropsy, both kidneys were harvested. Three portions of cortex tissues (~50 mg each) were collected, immediately flash frozen in liquid nitrogen, and stored at −75 ± 15 °C for quantitative real-time PCR of mRNA. The right kidney and remaining left kidney tissue were fixed in 10% buffer formalin, decalcified in formic acid after dehydration and clearing, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin. According to the well-established criteria (Kelleher et al., 1987; Yamamoto et al., 2007), morphological evaluations were firstly performed by a certified veterinary pathologist who was blinded to the animal treatments and biomarker data. Blind peer review was conducted by another certified pathologist. Histopathological grading of lesions was scored as none, minimal, mild, moderate, marked, and severe.

Quantitative real-time PCR. Total RNA was isolated from frozen kidney tissue using an RNAiso Plus Kit (Takara, Dalian) according to the manufacturer's instructions. The concentration of total RNA was measured using a Nanodrop spectrophotometer (Thermo, USA). Isolated RNA was reverse-transcribed with a High Capacity RNA-to-cDNA Kit (Applied Biosystems [ABI], USA). The TaqMan Gene Expression Assays (ABI) were used for determination of mRNA expression of *clusterin* and *NGAL*. Real-time PCR was performed using an ABI 7000 Real-Time PCR System with 20-μL reactions containing 1 μL of cDNA, 1 μL of each primer and probe, and 10 μL TaqMan Gene Expression Master Mix. Amplification was carried out using the following temperature profile: 10 min enzyme activation at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. To control for variation in the amount of cDNA available for real-time PCR in the different samples, mRNA expression levels of target sequences were normalized to the expression of an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta}$ method. The primer sequences for GAPDH were as follows: 5′-CCCCAATGTATCAGTTGTGGATCTG-3′ (forward) and 5′-CCTGCT TCACTACCTTCTTGATGTC-3′ (reverse), and the fluorescence probe was 5′-FAM-AAAGCTGCCAAATATG-3′. Results were presented as the mean fold change in mRNA expression compared to that in control animals ($n = 5$ animals per group).

Statistical analysis. The concentrations and/or activities of urinary biomarkers were normalized to the urinary Cr concentration. All data were represented as the mean ± standard deviation (SD). The distribution of all continuous parameters (normal versus non-normal) was assessed using Shapiro–Wilk's test, and differences between treated versus control animals were evaluated by Student's *t*-tests using SPSS 20.0 software (SPSS, USA). Graphs were plotted using the GraphPad Prism 5 software (GraphPad, USA). Receiver operator characteristics (ROC) analysis was performed as previously described using Statistical softwares SPSS 20.0 and MedCalc 12.7.7.0 (MedCalc, Belgium) (Dieterle et al., 2010; Han et al., 2008; Harpur et al., 2011). In brief, we first matched the biomarker data of all animals with corresponding histopathological findings at all 3 sampling points. Due to the spontaneous degeneration/regeneration of tubular cells in the control group, this histopathological finding was excluded in ROC analysis. ROC curves were plotted by entering data obtained from animals with renal tubule necrosis or tubular cell hyaline droplet formation versus the control and treated animals without histopathological changes

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